Reference: *Biol. Bull.* **170:** 267–278. (April, 1986)

EVIDENCE FOR HETEROTROPHY BY ZOOXANTHELLAE IN SYMBIOSIS WITH *AIPTASIA PULCHELLA*

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ABSTRACT

Sea anemones (*Aiptasia pulchella*) fed ^{35}S methionine labeled food translocated labeled material to symbiotic zooxanthellae. This uptake of host-derived organic materials by the symbiotic algae is interpreted as evidence for heterotrophy. It is calculated that 2.8–6.4% of the zooxanthellae growth requirement for protein was satisfied by heterotrophy under a 12 hour light:12 hour dark photoperiod. The cost of algal heterotrophy to the animal host was demonstrated by exposing aposymbiotic anemones to cultured zooxanthellae. Aposymbiotic animals rapidly re-established a stable symbiosis, but the symbiotic animals had a protein biomass significantly lower than control aposymbionts, suggesting that the reinfection of the symbiotic animals by zooxanthellae caused a decrease in animal biomass. Zooxanthellae spontaneously appeared and grew in the control aposymbionts. These repopulating algal cells were apparently derived from a residual heterotrophic population of zooxanthellae maintained by anemones after four years of darkness. Aposymbiotic anemones starved in darkness suffered a lower mortality rate than symbiotic animals under the same conditions, suggesting that heterotrophic symbiotic zooxanthellae can impose a fatal metabolic burden on these anemones.

INTRODUCTION

The metabolic relationship between intracellular dinoflagellate symbionts (= zooxanthellae; *Symbiodinium microadriaticum*) and their cnidarian hosts has been investigated extensively from the perspective of benefit of the algae to the animal. Zooxanthellae enhance the survival of symbiotic animals relative to that of aposymbiotic animals when both are starved in light (Fitt and Pardy, 1981). Zooxanthellae within host cells are photosynthetic, and tracer experiments have demonstrated that ^{14}C labeled algal photosynthate is translocated to the animal cells, where it is respired or incorporated into host proteins and lipids (Trench, 1981; Battey and Patton, 1984). Translocated carbon can potentially supply 20–150% of the carbon required for animal respiration (Muscatine *et al.*, 1983; McCloskey and Muscatine, 1984; Steen and Muscatine, 1984).

Little attention has been given to the possible benefit of symbiosis to the alga or the cost of symbiosis to the animal partner. Recently Douglas and Smith (1983) found that symbiotic hydra grew more slowly in darkness than aposymbiotic animals when both groups were given equal food rations, suggesting that zoochlorellae constitute a metabolic cost to the host in darkness. Several studies suggest that symbiotic algae are capable of uptake of organic material derived from the host. For example, Cook (1971) fed ^{35}S methionine labeled food to *Aiptasia* sp. and demonstrated that radioactively

Received 30 October 1985; accepted 14 January 1986.

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labeled substrates moved from host to algae, however the quantitative significance of this "reverse translocation" into the symbiotic algae was not assessed. Thorington and Margulis (1981) showed that when *Hydra viridis* was fed *Artemia* nauplii labeled with tritiated precursors of protein and nucleic acids, labeled tritium appeared in algal and animal fractions in a manner suggesting that algae and host might be competing for limited nutrients. However tritium is probably an unsuitable radionuclide for this type of study because proton exchange reactions are ubiquitous in living systems (Zubay, 1983). Cook (1983) has noted that ^{14}C would also be an inappropriate radiolabel for a reverse translocation study because of the possible metabolism of ^{14}C labeled organic substrates by the animal and fixation of the resulting $^{14}CO_2$ by algal photosynthesis.

In vitro experiments suggest that cultured zooxanthellae algae are capable of uptake of organic compounds potentially present in the host (Steen, 1985). Zooxanthellae freshly isolated from the anemone *Cassiopea xamachana* have an active transport mechanism for alanine uptake which is inhibited by a protein fraction present in host homogenate (Carroll and Blanquet, 1984). Cultured zooxanthellae from *Tridacna maxima* have active transport mechanisms for cysteine, methionine, and taurine (Deane and O'Brien, 1981).

These previous studies suggest that growth of symbiotic zooxanthellae is potentially augmented through uptake of host-derived organic compounds. This paper addresses the questions of whether algal heterotrophy occurs in symbiosis and whether the animal host incurs a metabolic cost from the maintenance of a population of symbiotic zooxanthellae.

I report evidence that symbiotic algae in a sub-tropical sea anemone can grow through heterotrophic uptake of host-derived organic materials even in light. Anemones with a rapidly growing population of zooxanthellae have lower protein biomass than normal aposymbiotic anemones, suggesting that the host incurs a metabolic cost from algal growth. Consistent with this, I report that symbiotic anemones starved in darkness have a higher rate of mortality than aposymbiotic anemones starved under the same conditions.

Materials and Methods

Animal maintenance

One clone of sea anemones (*Aiptasia pulchella*) derived from a single animal originally collected in Kaneohe Bay, Hawaii, by G. Muller-Parker, was maintained in a 10 gallon Plexiglas tank of aerated seawater at room temperature (20–25°C) exposed to indirect sunlight (50–100 $\mu Ein \cdot m^{-2} \cdot s^{-1}$). Animals were fed *Artemia* nauplii to repletion twice weekly and the tank was cleaned monthly. Anemones used in experiments were randomly selected, then segregated into several one liter finger bowls in 800 ml of seawater. Animals were starved for 3–4 days before use in experiments. During experiments, anemones were maintained in a controlled temperature incubator at 25°C ± 1° under 70 $\mu Ein \cdot m^{-2} \cdot s^{-1}$ irradiance (12 hour light:12 hour dark photoperiod) except as noted. Bowls of animals maintained under high irradiance were placed in a 25°C Lauda Brinkmann circulating water bath under a 500W Sylvania tungsten halogen "very wide flood" lamp that provided 300 $\mu Ein \cdot m^{-2} \cdot s^{-1}$ of light incident on the animals.

Aposymbiotic anemones were obtained by "cold stripping": normal symbiotic anemones were induced to extrude 99% of their symbiotic algae within one week by exposure to 4°C for 4 hours and subsequent maintenance in darkness. This technique

produces no animal mortality (Steen and Muscatine, in prep.). Some experiments used aposymbiotic anemones produced by prolonged (>4 years) culture in the dark.

Labeling animal food with ^{35}S methionine

A stock of 0.1 ml of 10 mCi · ml^{-1} ^{35}S-methionine in ethanol (New England Nuclear) was diluted to 1 ml with rat Ringers solution and the entire quantity was injected into the tail vein of an anesthetized nine month old female Sprague-Dawley rat. After three days the rat was sacrificed, and the internal organs (heart, liver, lungs, kidneys, and intestines) were removed and assayed for tissue specific activity (radioactive disintegrations · $minute^{-1}$ · mg $protein^{-1}$). Protein was measured by the method of Lowry *et al.* (1951). The intestines were the most heavily labeled tissue and were therefore used in all feeding experiments. Intestines were stored at −20°C until used.

The percent of total rat intestine ^{35}S-methionine in protein (rather than in intracellular free amino acid pools) was determined from sequential hot water extractions of rat intestine. About 65% of total radioactivity was hot water soluble, with 70% of the extractable counts removed during the first extraction. More extensive extraction led to tissue disintegration and a preparation unsuitable for feeding anemones. Therefore intestine was extracted only once in hot water, so that, of the total ^{35}S intestine fed to anemones, approximately 65% was particulate, with the remainder being unextracted soluble ^{35}S.

Translocation of ^{35}S-labeled compounds from host to algae

Four random samples of symbiotic anemones were segregated from a single population, and one of these groups was rendered aposymbiotic by "cold stripping." Each anemone was then individually fed a single 5–6 mm^2 piece of flattened rat intestine previously extracted once with hot water. Animals were allowed two hours in light for ingestion. Uningested food was then removed and the water in the bowls was replaced with fresh sea water before animals were placed in the incubator. Seawater was replaced initially after 4–6 hours to remove freshly egested food, then water was replaced daily in the animal bowls. After three days animals were individually homogenized in 10 ml of seawater in a glass tissue grinder with a motor-driven Teflon pestle. Zooxanthellae were separated by centrifugation for three minutes at $500 \times g$ in a Damon/IEC HN-S clinical centrifuge. One ml aliquots of animal homogenate were placed in scintillation vials, dried at low heat on a hot plate, and solubilized in toluene-Triton X scintillation fluor. The algal pellets were washed three times by resuspension and centrifugation in seawater, then placed in a scintillation vial, and dried. Ten ml of fluor was added to the dried remains and the vials were stored overnight in darkness before scintillation counting. Radioactivity was measured in a Beckman LS 100C scintillation counter and all counts were background and quench corrected. Radioactive disintegrations per minute (dpm) for each sample were calculated with ^{35}S half-life corrections. The same procedure was also followed using aposymbiotic anemones.

Reinfection of aposymbiotic animals with cultured zooxanthellae

Anemones which had been rendered aposymbiotic by prolonged (>4 years) maintenance in darkness, were randomly assigned to two groups of 12 animals each. One group was exposed to a 1 ml innoculum of 10^6 cultured zooxanthellae (CZ) cells (Steen, in prep.), washed and resuspended in Millipore-filtered (0.45 μ pore size) seawater, then added to the bowl in which animals were maintained. The other group

was incubated without exposure to CZ. Both groups were maintained in Millipore-filtered seawater which was changed daily. A third group of similar size light-reared symbiotic anemones was segregated and all three groups were maintained in an incubator at 56 $\mu Ein \cdot m^{-2} \cdot s^{-1}$ for 17 days. Animals were then individually homogenized and the zooxanthellae separated as before. Anemone biomass was measured as animal protein by the Lowry technique (1951) and number of zooxanthellae was normalized to animal protein. Algal mitotic index was measured (Wilkerson *et al.*, 1983) at 430× on a hemocytometer slide using an Olympus BH-2 microscope and cell specific growth rate was calculated after McDuff and Chisholm (1982).

Survival of starved animals

Twenty-five individuals each of symbiotic and aposymbiotic anemones were randomly selected and placed in 1 liter finger bowls. Both groups of anemones were starved in darkness at 25°C. Seawater was changed twice weekly and animals were censused at the same time. Dead animals were seldom observed, because animals shrank during starvation and animals tended to dissolve upon death. The experiment was terminated at week 15 and the "symbiotic" anemones were individually homogenized, the zooxanthellae collected by centrifugation, and the number of zooxanthellae per anemone calculated from hemocytometer counts of aliquots of the resuspended zooxanthellae.

RESULTS

Translocation of ^{35}S label from host to algae

Direct evidence for "reverse translocation" was obtained by feeding symbiotic animals with radioactive food and measuring the subsequent appearance of label in the zooxanthellae. The average total dpm of food given to each anemone was 9014.4 ± 1957.7 dpm. The specific activity of the food was 486.7 dpm per 1 μg protein (see Discussion), so the average meal was 18.5 μg protein.

The average assimilation of ^{35}S-methionine label into all groups of symbiotic animals after three days averaged 21.6% (Table I), calculated by dividing dpm assimilated by the average activity of ingested food particles. Since anemones had been randomly

TABLE I

Assimilation of ^{35}S label by Aiptasia pulchella *and symbiotic zooxanthellae after a three day incubation*

	Total ^{35}S dpm assimilated per anemone	Average assimilation efficiency (%)	Percent total assimilated dpm in algal pellet
Constant light	1771.3 (±968.6)	19.67	10.17 (±0.16)
Constant darkness	2264.4 (±1079.1)	25.07	10.82 (±0.22)
Light/dark photoperiod	1810.6 (±865.6)	20.09	12.26 (±0.37)
Aposymbiotic anemones (darkness)	3859.8 (±1568.1)	42.82	6.53 (±0.16)

Animals were fed pieces of ^{35}S-methionine labeled rat intestinal tissue. Incubator irradiance was 70 $\mu Ein \cdot m^{-2} \cdot s^{-1}$ in the light experiments. All animals were symbiotic except the aposymbiotic dark control. Activity is shown as disintegrations · min^{-1} (dpm) ± standard deviation. Results shown are pooled from two separate experiments with a total sample size of n = 27 animals in every case except the experiment with aposymbiotic anemones where n = 15. Pellet-associated radioactivity is ^{35}S dpm in zooxanthellae and/or in animal tissue contaminants of the algal pellet.

selected from a single population, it is assumed that the average animal biomass of each group of anemones was equivalent. Therefore the extent of ingestion and assimilation of ^{35}S methionine label differs between groups because of the treatments, not because of anemone size differences. Dark-maintained animals assimilated significantly more radioactive label than did animals in constant light or on a 12 light:12 dark photoperiod (t-test; $P < 0.05$). Aposymbiotic animals assimilated significantly more label than did any of the symbiotic animal groups (t-test; $P < 0.01$). It was observed that aposymbiotic animals held food in the gut for as long as 24 hours, while symbiotic anemones tended to egest food within 6 hours.

In constant light and constant darkness appearance of dpm in the algal pellet was 10.2% and 10.8%, respectively, of total dpm assimilated, while the appearance of dpm in the zooxanthellae of anemones on a light/dark photoperiod was 12.3%. Since pellet-associated ^{35}S could arise from ^{35}S labeled animal tissue contaminants as well as algal assimilation of ^{35}S methionine, the radioactivity in the aposymbiotic animal pellet was used as a control for pellet contaminants of animal origin. The percentage of pellet-associated radioactivity was significantly lower in aposymbiotic than in all groups of symbiotic animals (arcsine transformed data used for t-test; $P < 0.0005$) (Zar, 1974).

The largest proportion of total activity in the algal pellet was found in animals on a light/dark photoperiod (arcsine transformed data used for t-test; $P < 0.025$). After correction for animal tissue contaminants in the algal pellet, the proportion of zooxanthellae-associated ^{35}S-methionine radioactivity was 3.6%, 4.3%, and 5.7% in anemones maintained in constant light, constant darkness, or on a photoperiod, respectively. Movement of ^{35}S label into the algal pellet is interpreted as heterotrophic uptake by the zooxanthellae. However the measured amount of ^{35}S label in zooxanthellae isolated from dark-maintained anemones may underestimate the extent of algal heterotrophy in this case, because *A. pulchella* rapidly extrude pellets of symbiotic zooxanthellae in darkness (Steen and Muscatine, in prep.). Results presented here do not exclude the possibility that such pellets were radioactively labeled, leading to an underestimate of the total extent of ^{35}S incorporation into zooxanthellae symbiotic with dark-maintained hosts.

Reinfection of aposymbiotic animals

To assess cost to the host of a growing population of symbiotic algae, aposymbiotic animals were reinfected with CZ. *A. pulchella* were fed twice weekly in darkness for four years at which time microscopic examination revealed no pigmented cells with recognizable zooxanthellae morphology. Seventeen days after innoculation with CZ these animals re-established the relationship so that no significant differences were observed between symbiotic (control) and reinfected animals in numbers of zooxanthellae per anemone or zooxanthellae density (Table II). However both standing stock and density of zooxanthellae were significantly higher in reinfected animals than in light-maintained aposymbiotic control animals which did not receive an inoculum of CZ (Wilcoxon two-sample test; $P < 0.001$). Therefore CZ were able to repopulate aposymbiotic hosts and to reach densities comparable to zooxanthellae in normal light-reared animals.

Survival of starved animals

Aposymbiotic and symbiotic animals were starved in darkness to ascertain whether and to what extent the presence of potentially heterotrophic symbiotic algae affected the survival of the host. Figure 1 shows that, during the 15 week starvation period,

TABLE II

Reinfection of aposymbiotic Aiptasia pulchella *with cultured zooxanthellae*

	Anemone biomass 17 days after exposure to CZ (mg protein · anemone^{-1})	Zooxanthellae per anemone ($\times 10^5$)	Zooxanthellae cell density (10^5 · mg animal prot^{-1})	Algal mitotic index (% dividing cells)	Cell specific growth rate (day^{-1})
Uninoculated apo control anemones n = 12	1.039 (±0.22)*	1.11 (±1.78)*	0.94 (±1.35)*	—	—
Inoculated apo anemones n = 12	0.623 (±0.26)	5.82 (±3.55) ┐ NS	10.41 (±6.87) ┐ NS	1.05 (±0.22)	0.0514
Light-reared sym anemones n = 12	0.885 (±0.37)	7.72 (±6.07) ┘	7.87 (±3.61) ┘	0.85 (±0.15)	0.0416

* Includes four anemones which spontaneously reinfected with zooxanthellae to levels comparable to sym anemones.

Aposymbiotic (apo) animals were maintained for 17 days in Millipore-filtered (0.45 μ) seawater changed daily at 25° ± 1°C under an irradiance of 56 μEin · m^{-2} · s^{-1}. Aposymbiotic animals were obtained from G. Muller-Parker and the control group was comprised of similar size light-reared symbiotic (sym) anemones. Animals were individually homogenized at the end of 17 days and zooxanthellae were separated by centrifugation. Anemone biomass was measured as animal Lowry protein and zooxanthellae per anemone is expressed as the number of cells per mg animal protein. Algal mitotic index was measured from 10 samples of 100 zooxanthellae each. Values shown are mean ± standard deviation. NS indicates no significant difference between values.

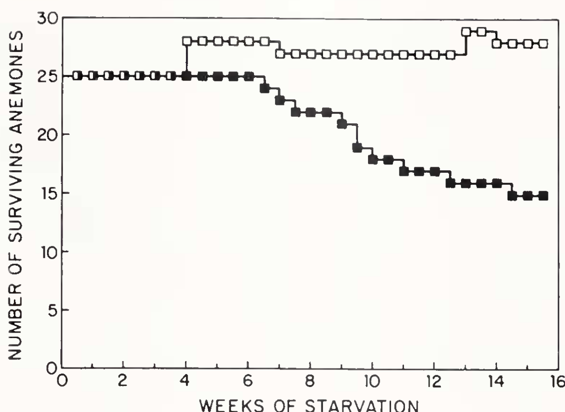

FIGURE 1. Survival of dark-maintained starved symbiotic (■) and aposymbiotic (□) *A. pulchella*. The aposymbiotic animal population underwent a slight increase because of the production of new pedal lacerates. Symbiotic animals progressively declined in number after week 6.

aposymbiotic animals starved in darkness produced pedal lacerates thereby increasing the total number of individuals present. The death of a single aposymbiotic anemone at week 7 was a newly produced pedal lacerate. No further deaths occurred in the aposymbiotic population until week 14, when another new pedal lacerate died. Although no quantitative data is available, the aposymbiotic animals appeared to be larger at the end of the experiment than the symbiotic animals.

The survival of symbiotic animals starved in darkness was significantly lower than that of aposymbionts (log-rank method adapted from Azen *et al.*, 1977; $\chi^2 = 12.27$; $P < 0.001$). No pedal lacerates were produced and the first death (disappearance) of a symbiotic animal occurred at week 6. After week 6 symbiotic animals died at the rate of more than one a week for 6 weeks. During this period of starvation symbiotic animals progressively lost symbiotic algae. The overall percentage of survival at week 15 was 60% for symbiotic anemones and 112% for aposymbionts. The experiment was terminated at week 15 because most of the surviving symbiotic animals were depleted of zooxanthellae. The average number of zooxanthellae per anemone in the symbiotic animals at the end of the experiment was 0.17×10^5, 98% fewer zooxanthellae than a similar light-reared control (Table II).

DISCUSSION

Reverse translocation

The ^{35}S experiments suggest that uptake of host-derived organic materials by zooxanthellae occurs in laboratory-maintained *A. pulchella*. Cook (1971) first used ^{35}S-methionine labeled protein to show movement of radiolabel from *Aiptasia* sp. into symbiotic zooxanthellae. However the magnitude of reverse translocation reported (Cook, 1971) was higher and more variable than reported here. Five days after feeding *Aiptasia* sp. with ^{35}S labeled food, approximately 31% (±10% standard deviation) of the total recovered radioactivity was associated with the algal pellet (Cook, 1971). However no indication was given of the extent of animal tissue contamination in the algal pellet. In the present study, such contamination was observed to be greater than 50% of the pellet-associated radioactivity.

The average assimilation efficiency of ^{35}S methionine-labeled protein by *A. pulchella* was 21.61% (Table I). This figure is low compared to the 61% digestive assimilation efficiency of *A. pulchella* calculated from the ratio of caloric loss in feces to caloric intake in food (Hunter, 1984). However this ratio does not include caloric loss in dissolved or microparticulate organic matter, so it is possible that assimilation efficiency was overestimated (Hunter, 1984). Preliminary results (Steen, unpub.) suggest that the average efficiency of assimilation of ^{35}S methionine dissolved in seawater into animal tissue is considerably less than 1%. This suggests that assimilation of ^{35}S methionine into anemone tissue was mainly assimilation of food protein rather than assimilation of pools of free methionine in the food particles.

The contribution of heterotrophy to algal growth (CHAG) was calculated using the data for reverse translocation of ^{35}S-methionine in animals on a 12L:12D photoperiod (Table I). Standing stock of algae in *A. pulchella* was 0.77×10^6 zooxanthellae per anemone (Table II; light reared control) and the cell specific growth rate for zooxanthellae within the host was calculated as $\mu = 0.0416 \cdot \text{day}^{-1}$ (Table II; light reared control) using $t_d = 4.88$ hours (Steen, 1985). The number of new zooxanthellae cells produced during three days of the experiment was;

$$\text{New cells} = 3\mu \times \text{standing stock of zooxanthellae}$$
$$= 9.61 \times 10^4 \text{ zooxanthellae}$$

Lowry protein assays of freshly isolated washed zooxanthellae showed there was 78.5 μg protein per 10^6 zooxanthellae. The product of the number of new cells and the protein per cell equals the quantity of new zooxanthellae protein synthesized during the experiment.

$$\text{New zooxanthellae protein} = 78.5 \times 0.0961$$
$$= 7.5439\ \mu\text{g protein} \quad (1)$$

The proportion of total radioactivity incorporated into zooxanthellae was calculated as 5.73%, the difference between the light:dark photoperiod algal pellet and the aposymbiont pellet (Table I). Therefore;

$$\text{Zooxanthellae-associated dpm} = \text{total dpm assimilated} \times 0.0573$$
$$= 103.7 \text{ dpm}$$

The specific activity of ^{35}S-methionine labeled rat intestine was 486.7 $\text{dpm} \cdot \mu\text{g protein}^{-1}$ (regression not shown; $r = 0.92$). Assuming that the specific activity of zooxanthellae protein was identical to the specific activity of food protein and further assuming that all ^{35}S in plant cells is incorporated into protein (*cf.*, Giovanelli *et al.*, 1980), it is possible to calculate the approximate net amount of ^{35}S-labeled zooxanthellae protein produced in three days.

$$^{35}\text{S-labeled protein synthesized} = 103.7/486.7$$
$$= 0.2131\ \mu\text{g} \quad (2)$$

The contribution of heterotrophy to algal growth is calculated as (2) divided by (1) or;

$$(^{35}\text{S protein/total new protein}) \times 100 = 2.82\%$$

Therefore almost 3% of zooxanthellae growth, assayed by protein synthesis was satisfied by reverse translocation of ^{35}S-methionine, although the irradiance permitted net algal photosynthesis (Muller-Parker, 1984). This estimate is likely to be conservative because the zooxanthellae growth rate was calculated assuming $t_d = 4.88$ hours (Steen,

1985). If the value used for duration of mitosis is $t_d = 11$ hours (Wilkerson *et al.*, 1983), the estimate of CHAG becomes 6.39%. This estimate of algal heterotrophy may be further biased by the assumption that all new zooxanthellae cells produced by mitosis are retained by the host, rather than being expelled. O. Hoegh-Guldberg (pers. comm.) has shown that the rate of expulsion of symbiotic zooxanthellae may be as high as 26% of the rate of production of new zooxanthellae by mitosis. If newly produced cells labeled with ^{35}S are expelled, this would lead to an underestimate of the extent of heterotrophy.

It is possible that the zooxanthellae in this experiment became ^{35}S labeled by uptake and assimilation of inorganic rather than organic sulfur. Cultured *Tridacna* zooxanthellae are capable of active transport of sulfate as well as cysteine, methionine, and taurine (Deane and O'Brien, 1981). However methionine, in plants and animals, is chiefly utilized as a component of protein, methionyl tRNA, or S-adenosyl-L-methionine (Giovanelli *et al.*, 1980). Moreover the oxidative metabolism of sulfur-containing amino acids to sulfate is slow (Sinensky, 1977), and is therefore not relevant to the present study.

About 90% of the total sulfur in plant tissue is in the amino acids methionine and cysteine, and nearly all of these amino acids are in protein (Giovanelli *et al.*, 1980). Thus it is reasonable to assume that most of the ^{35}S activity in zooxanthellae was incorporated into protein and that the specific activity of ^{35}S labeled zooxanthellae protein was roughly equivalent to the specific activity of food protein.

The data on zooxanthellae-associated ^{35}S (Table I) suggests that heterotrophic uptake and/or growth in darkness or on a photoperiod is stimulated relative to zooxanthellae maintained in constant light. Natural populations of marine and freshwater algae synthesize protein during darkness, and night time net protein synthesis is often nearly equal to protein synthesis in light (Cuhel *et al.*, 1984). Furthermore it has been demonstrated that sulfate incorporation into protein in algae measured net protein synthesis rather than turnover (Cuhel *et al.*, 1984), suggesting that ^{35}S methionine incorporation is a reasonable measure of cell growth. Cuhel *et al.* (1984) report that sulfate uptake by *Dunaliella* is only slightly stimulated by light, and that cultures of freshwater algae may incorporate sulfate more rapidly in darkness than in low light.

Cultures of the diatom *Phaeodactylum tricornutum* maintained under conditions of environmental stress (such as low irradiance levels) conserved the ability to synthesize protein more than the ability to synthesize lipids or polysaccharides (Morris *et al.*, 1974). This increased protein synthesis was generally at the expense of the algal polysaccharide fraction (Morris *et al.*, 1974). A similar phenomenon may have been occurring with zooxanthellae in symbiosis; perhaps the allocation of heterotrophically derived carbon to protein synthesis increases proportionally as the zooxanthellae become light limited. Since uptake of methionine by zooxanthellae is an active process (Deane and O'Brien, 1981), heterotrophic uptake in constant darkness may be less than that in cells maintained on a photoperiod because the zooxanthellae can become energy-limited in darkness.

Results presented here suggest that reverse translocation or algal heterotrophy is possible in an intact symbiosis. Heterotrophy was greatest when zooxanthellae were exposed to a photoperiod similar to that which symbiotic organisms experience in the field. However the animals in this experiment experienced an irradiance much lower than natural ambient light. Muller-Parker (1984) found that the compensation irradiance of intact *A. pulchella* was 32 $\mu Ein \cdot m^{-2} \cdot s^{-1}$, but that algal photosynthesis did not saturate below 400 $\mu Ein \cdot m^{-2} \cdot s^{-1}$. Experimental animals were irradiated with 70 $\mu Ein \cdot m^{-2} \cdot s^{-1}$, and thus experienced less than 20% of saturating irradiance (Muller-Parker, 1984). It is not known to what extent algal heterotrophy would be affected by ambient irradiance.

Reinfection of aposymbiotic animals

To determine if zooxanthellae within hosts can survive by heterotrophic uptake of host-derived organic material, I analyzed two independent sets of observations in the reinfection experiment. After the light incubation period one third (4 of 12) of the uninoculated aposymbiotic animals (Table II) contained recognizable zooxanthellae; these were probably derived from an undetected population of algae which had survived four years of darkness. Since animals had been fed in the dark it seems likely that zooxanthellae had survived by using materials from food ingested by the animal.

Table II also shows that the zooxanthellae which repopulated aposymbiotic animals were growing significantly more rapidly than zooxanthellae in normal light-reared anemones ($P < 0.01$) based on their mitotic index. This indicates that hosts may have become repopulated with zooxanthellae to a level comparable with normal symbiotic hosts through the rapid growth of zooxanthellae in the unexploited habitat presented by an aposymbiotic host. Reinfected animals had significantly lower protein biomass than aposymbiotic animals ($P < 0.005$) because the metabolic cost to the host may be particularly high when symbionts undergo a period of relatively rapid growth.

Survival of starved animals

The metabolic cost of zooxanthellae to a symbiotic animal is most clearly manifested in the survival curves for symbiotic and aposymbiotic animals (Fig. 1). Anemones were not weighed during this experiment, but the weight loss experienced by reinfected animals (Table II) suggests that the symbiotic animals died first because of the depletion of organic materials caused by zooxanthellae heterotrophy. These results are in contrast with data from *Anthopleura elegantissima;* normal symbiotic anemones starved in darkness lost proportionally less reduced weight than albino anemones starved in darkness (Muscatine, 1961; Fitt and Pardy, 1981).

The interaction between zooxanthellae and *A. pulchella* is dynamic, and may change in character from a commensalism or a mutualism to a parasitism depending upon environmental conditions. The dynamic nature of the relationship is demonstrated by the observation that *A. pulchella* is capable of expelling zooxanthellae in periods of prolonged darkness (Steen and Muscatine, in prep.), while zooxanthellae can grow rapidly in an unexploited host (Table II).

Ecological considerations

Caution must be used when comparing the results of studies on different symbiotic hosts, because it is likely that symbiotic zooxanthellae are composed of several strains or species (Blank and Trench, 1985). Consequently the relationship between different hosts and their symbionts may vary considerably. However if zooxanthellae are generally capable of facultative heterotrophy, how important is heterotrophy in the field? Corals or anemones growing at great depth or high latitude experience attenuated solar irradiance such that algae may be light limited. Dustan (1982) has even suggested zooxanthellae are normally light-limited in their natural habitat, as photosynthetic light saturation of isolated zooxanthellae always occurred at higher irradiance than was measured at the collection depth at local noon. The depth of penetrance of 1% of the total surface irradiance is generally accepted as the algal photosynthetic compensation depth, yet nine species of corals have been reported to maintain symbiotic zooxanthellae at great depths even though these zooxanthellae are seemingly unable to be phototrophic (Fricke and Schuhmacher, 1983). The symbiotic coral *Leptoseris fragilis* has been found at 145 m in the Red Sea, a depth to which only 0.17% of total surface irradiance penetrates (Fricke and Schuhmacher, 1983).

The ability of symbiotic zooxanthellae to tolerate extended periods of darkness is consistent with the hypothesis that zooxanthellae can survive by heterotrophic uptake of host derived organic substrates. Yonge and Nicholls (1931) reported that zooxanthellae of *Psammocora gonagra* appeared normal after 166 days of host starvation in darkness. Corals starved in darkness had 60% fewer zooxanthellae than normal corals, but these zooxanthellae were still capable of photosynthetic oxygen production (Yonge and Nicholls, 1931). Kevin and Hudson (1979) found that *Plesiastrea urvillei* maintained a normal complement of zooxanthellae during periods of total darkness for at least 48 days. Some zooxanthellae were still present after 150 days of darkness, and colonies which were bleached in darkness, then maintained in light, re-established the symbiosis (Kevin and Hudson, 1979). The recolonizing zooxanthellae were presumably derived from a population which had survived the period of darkness. Data reported here show regrowth of zooxanthellae in *Aiptasia pulchella* which had been maintained for four years in darkness, then brought into light. The reinfecting zooxanthellae were not derived from free-living populations since animals were maintained in Millipore-filtered (0.45 μ) seawater.

The results presented here suggest that laboratory maintained *A. pulchella* may incur a metabolic cost from having symbiotic algae, due to an algal requirement for heterotrophic substrates some or all of the time. However the benefit to the host from symbiosis may not lie in the photosynthetically fixed carbon which is thought to be translocated from algae to host. *In vivo* ^{31}P nuclear magnetic resonance analysis of symbiotic *A. pulchella* has shown that photosynthesis by symbiotic zooxanthellae does not significantly affect the bioenergetic status of the anemone host (Steen, 1985). Perhaps the advantage of having symbiotic zooxanthellae lies in their ability to produce oxygen in photosynthesis (Fredericks, 1976; Rinkevich and Loya, 1984), or to synthesize some organic material ordinarily limiting to the animal (Von Holt, 1968; Kokke *et al.*, 1981).

Acknowledgments

Supported by a UCLA Chancellors Intern Fellowship. I thank A. Durstenfeld for performing the rat tail vein injection, and L. Muscatine, K. Dunn, J. Smith, and W. O'Loughlin for discussion and criticism.

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